Effects of the ruminal comminution rate and microbial contamination of particles on accuracy of *in situ* estimates of ruminal degradability and intestinal digestibility of feedstuffs

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Keywords
effective degradability, intestinal effective digestibility, microbial contamination, protein, ruminal transit

Summary

Effects of considering the comminution rate (k_c) and the correction of microbial contamination (using ^{15}N techniques) of particles in the rumen on estimates of ruminally undegraded fractions and their intestinal digestibility were examined generating composite samples (from rumen-incubated residues) representative of the undegraded feed rumen outflow. The study used sunflower meal (SFM) and Italian ryegrass hay (RGH) and three rumen and duodenum cannulated wethers fed with a 40:60 RGH to concentrate diet (75 g DM/kgBW$^{0.75}$). Transit studies up to the duodenum with Yb-SFM and Eu-RGH marked samples showed higher k_c values (/h) in SFM than in RGH (0.577 vs. 0.0892, p = 0.034), whereas similar values occurred for the rumen passage rate (k_p). Estimates of ruminally undegraded and intestinal digestibility of all tested fractions decreased when k_c was considered and also applying microbial correction. Thus, microbial uncorrected k_p-based proportions of intestinal digested undegraded crude protein overestimated those corrected and k_c–k_p-based by 39% in SFM (0.146 vs. 0.105) and 761% in RGH (0.373 vs. 0.0433). Results show that both k_c and microbial contamination correction should be considered to obtain accurate *in situ* estimates in grasses, whereas in protein concentrates not considering k_c is an important source of error.

Introduction

Progress in the development of current protein evaluation systems for ruminants requires accurate effective estimates of protein ruminally undegraded and intestinally digested of feeds. However, the *in situ* methodology employed in many studies is poorly adapted to the complex digestive physiology of the ruminant and too simplistic because (i) the rate of comminution and mixing of particles in the rumen (k_c) is not considered in addition to its rumen passage rate (k_p) to determine the ruminally undegraded feed fraction and (ii) the protein intestinal digestibility is often determined on the residues of only one or few rumen incubation times despite that this value decreases with increasing rumen residence times in most feedstuffs (De Boer et al., 1987; Yang and Poncet, 1988; González et al., 1999). This decrease is a consequence of the progressive increase in intestinal undigestible materials in feed particles with the extent of rumen degradation with time (González et al., 1999). Therefore, intestinal effective digestibility (IED) estimates corresponding to the digestibility of the total flow from the rumen of undegraded protein are needed. Also, errors introduced by the contamination of rumen-incubated residues with adherent micro-organisms limit the accuracy of the *in situ* techniques. The overestimation of crude

protein (CP) ruminally undegraded (RUCP) by not correcting this contamination varies considerably among feeds, being especially important in those with low CP and high fibre contents (Michalet-Doreau and Ould-Bah, 1989; Rodríguez and González, 2006). In addition, as adherent micro-organisms are digested in the intestine, such contamination may also lead to overestimations of intestinal digestibility (González et al., 2009). The lack of consideration of k_c also leads to overestimations of RUCP (González et al., 2006b). Also, as proportional microbial contamination raises with rumen residence time (Bernard et al., 1988; Rodríguez and González, 2006), the increase in this time associated with the consideration of k_c may also affect the accuracy of microbial contamination estimates. González et al. (1999) proposed a method to estimate IED based on the integration of functions which describe the variations of intestinal digestibility and undegraded feed outflow with the residence time of feed particles in the rumen; however, this is a very complex and laborious procedure. To facilitate the determination of the ruminally undegraded feed fraction and its IED, González et al. (2009), using only k_p, proposed an *in situ* approach based on generating a sample representative of the chemical composition of the rumen-undegraded feed flow pooling the different residues incubated in the rumen at increased times. The aims of this work were to develop a similar model taking into account k_c and to examine the importance of the lack of both k_c in the model and microbial contamination correction on estimates of ruminally undegraded fractions and IED of two feeds with very different physical, chemical and degradative characteristics, being sunflower extraction meal (SFM), representing a highly degradable concentrate, and Italian ryegrass hay (RGH), representing a low-quality grass.

Materials and methods

Tested feeds, animals and feeding

The SFM sample was obtained by solvent extraction from semi-dehulled seeds, whereas that of RGH corresponds to a late first cut sun cured in fine weather. Both samples were ground to pass a 2-mm screen for *in situ* studies and to pass a 1-mm screen for chemical analysis (Table 1). Three wethers (3 years old; 76.3 kg BW as mean) fitted with rumen cannulae (inside diameter 60 mm) and *T*-type duodenal cannulae (inside diameter 12 mm) were used to estimate non-corrected and corrected (for feed microbial contamination in the rumen) values of both ruminally undegraded fractions and IED. The diet had a 40:60 RGH to concentrate ratio and included both tested feeds. The RGH was chopped at 8–10 cm. The concentrate contained (g/kg), in addition to SFM (200), wheat grain (790), $CaCO_3$ (4.0), NaCl (3.0) and a premix of trace minerals and vitamins (3.0). This diet contained (per kg DM) 127 g CP and 362 g neutral detergent fibre. The diet was fed starting 10 days before the experimental period, at 75 g DM per kg metabolic weight in six equal meals (every 4 h), starting at 0900 h. The energy supply of this diet represents 2.3 times the maintenance requirements. Wethers were housed in individual pens and handled according to the animal care principles as published in the Spanish Royal Decree 1201/2005 (B.O.E., 2005).

Table 1 Chemical composition (g/kg DM) of tested feeds

Item	SFM	RGH
Crude protein	363	63.5
Ash	67.1	81.7
Ether extract	9.30	15.7
Neutral detergent fibre	449	584
Acid detergent fibre	297	351
Acid detergent lignin	84.3	39.8
NDIN	9.00	3.59
ADIN	3.64	1.36

SFM, sunflower meal; RGH: Italian ryegrass hay; NDIN, insoluble N in neutral detergent solution; ADIN, insoluble N in acid detergent solution.
Values are means of three replicates.

Experimental procedures

Transit through the forestomachs of RGH and SFM particles was determined by pulse dosing (30 min before the 0900-h meal) each animal with 40 g of both rare earth-labelled feeds, which were normally consumed in 20 min. Previously, the feeds were sewn into bags of the same nylon tissue used in the incubations (46 μm of pore size), washed with a commercial detergent (based on sodium lauryl sulphate) through the laundering cycle of an automatic washer to eliminate its soluble components (Udén et al., 1980) and oven-dried at 60 °C for 48 h. Residues were then labelled by immersion in ytterbium (Yb; SFM) or europium (Eu; RGH) solutions using the procedure indicated by González et al. (1998) at a dose of 10 mg rare earth/g of feed residue. Feed particle size was not altered in the labelling process. A total of 23 samples were obtained from the duodenal cannula, the first before feeding the marked feeds

and the rest at intervals of 1.5, 3, 4, 6 and 12 h in the periods of 0 to 15, 15 to 24, 24 to 36, 36 to 60 and 60 to 84 h post-dosage respectively. These samples were oven-dried at 105 °C for 48 h, milled to pass a 1-mm screen and analysed for Yb and Eu. Values of k_p and k_c were determined by fitting by wether the evolution with time of the marker concentration in duodenal digesta to the model of Grovum and Williams (1973) by non-linear regression. In agreement with Ellis et al. (1979) and González et al. (2006b), primary and secondary rate constants of this model were assumed to be k_p and k_c respectively.

Nylon bags (110 × 70 mm inner dimensions) of the same nylon tissue were filled with approximately 3 g (air dry) of the samples ground to pass a 2-mm screen. Two incubation series with duplicate bags were performed for each feed and wether. The incubation schedule was established to assure the asymptotic convergence of values using six incubation times. Thus, bags filled with SFM were incubated in the rumen for 2, 4, 8, 16, 24 and 48 h and those filled with RGH were incubated for 3, 6, 12, 24, 48 and 72 h. In each series, all bags were simultaneously placed in the rumen just before feeding the 0900-h meal. After being collected from the rumen, bags were washed with tap water and stored at −20 °C. After thawing, bags were washed three times for 5 min in a turbine washing machine (Jata 580; JATA, Zubibitarte; 8 – 48220 Abadiano, Bizkaia, Spain). The same procedure was applied to two series of two bags to obtain the 0-h value. For each wether and incubation time, one bag of each incubation series was oven-dried for 48 h at 80 °C and analysed for DM and N to establish the respective ruminal degradation kinetics. The other bag was stored at −20 °C, freeze-dried and used to generate, for each wether, a composite sample representative of the rumen-undegraded DM flow. These samples were used to determine non-corrected and corrected values of DM and organic matter (OM) ruminally undegraded (RUDM and RUOM), RUCP and of IED of DM and CP. To estimate RUDM, RUOM and RUCP, the composite samples were analysed for these fractions. To determine IED, eight subsamples with approximately 200 mg of each composite sample were weighed into mobile nylon bags with an approximately round shape (∅ ≈ 3 cm). These bags were introduced randomly at a rate of one bag every 15 min (eight bags per wether per day) through the duodenal cannula and recovered from faeces. They were then stored at −20 °C. After thawing, they were washed as described for the ruminally incubated bags, oven-dried at 80 °C for 48 h and weighed to determine the IED of DM. Finally, these residues were pooled by wether prior to N analysis. Ruminal and intestinal digestive estimates were completed considering only k_p or k_p and k_c together.

Contamination owing to rumen micro-organisms in ruminally and intestinally incubated residues was determined by labelling rumen micro-organisms with ^{15}N by a continuous intrarumen infusion of ammonium sulphate (30 mg ^{15}N /day, 98 atoms % enriched) from 5 days before the start of the incubation period to its end. Then, before stopping the infusion, representative samples of rumen contents were obtained to isolate solid-associated bacteria (SAB) as described by Rodríguez et al. (2000). Isolated SAB samples were lyophilized and analysed for DM, N and $^{15}N/N$. Microbial proportions of N and DM of the rumen-undegraded or intestinal digested samples were determined as indicated by González et al. (1998). Microbial proportions of OM in rumen-undegraded composite samples were determined as microbial DM × (OM in SAB/OM in residue).

To evaluate in both feeds the possible losses of particles from the bags during rumen incubation, solubility of DM was determined by incubating samples (0.55 g in quadruplicate) in 40 ml McDougall buffer (pH = 6.8) at room temperature (i.e. 22 °C) for 1 h in a shaking water bath. After filtration through 7- to 9-μm pore size paper discs (Filter Lab no 1242; Filtros ANOIA, SA, Barcelona, Spain), solid residues were dried (80 °C for 48 h) and weighed to determine DM solubility.

Calculations and generation of rumen-undegraded representative samples

The disappearance data of DM or CP were fitted for each wether according to the exponential model of Ørskov and McDonald (1979) as:

$$d = a + b \cdot \left(1 - e^{-k_d t}\right) \quad (1)$$

In this model, constants 'a' and 'b' represent, respectively, the soluble fraction and the non-soluble but degradable fraction which disappears at a constant fractional rate, k_d, per unit time. The undegradable fraction (r) was estimated as proportion as 1 – (a + b).

Estimates of RUDM or RUCP with the integration method were obtained as the complementary value of the ruminal effective degradability calculated by the equations proposed by Ørskov and McDonald (1979) (equation 2) and McDonald (cited by the ARC, 1984) (equation 3) using one- or two-pool models respectively:

$$\text{RUDM or RUCP} = 1 - \left(a + b \cdot \left(\frac{k_d}{k_d + k_p}\right)\right) \quad (2)$$

$$\text{RUDM or RUCP} = 1 - \left(a + b \cdot \left(\frac{k_d}{k_d + k_p}\right) \cdot \left(\frac{k_d + k_p + k_c}{k_d + k_c}\right)\right) \quad (3)$$

To obtain the composite samples of ruminally undegraded residues representative of rumen outflow of undegraded DM used in the proposed method, the freeze-dried residues from both incubation series of each animal were pooled in equal quantities for each incubation time. Then, these last samples were mixed in predetermined proportions. Thus, in SFM, the rumen-incubated residues at 0, 2, 4, 8, 16, 24 and 48 h were considered representative of the particle feed rumen outflow for the intervals of 0 to 1, 1 to 3, 3 to 6, 6 to 12, 12 to 20, 20 to 36, and 36 to 60 h, respectively, whereas in RGH, the residues at 0, 3, 6, 12, 24, 48 and 72 h were considered representative for the intervals of 0 to 1.5, 1.5 to 4.5, 4.5 to 9, 9 to 18, 18 to 36, 36 to 60 and 60 to 84 h respectively. When only k_p was considered, the rumen outflow of feed DM ($\varnothing$) in these intervals was calculated, as indicated by González et al. (1999), as:

$$\varnothing_{\text{until}\,t} = r(1 - e^{-k_p t}) + \frac{bk_p}{k_d + k_p}(1 - e^{-(k_d + k_p)^t}) \quad (4)$$

In accordance with the method of these authors, when k_c and k_p are used together, rumen outflow of feed DM can be described considering that undegraded material is defined by $u = r + b\ e^{-kdt}$, and that rumen outflow is defined by $f = 1 - [(k_c e^{-kpt} - k_p e^{-kct})/(k_c - k_p)]$. Thus, the corrected outflow rate from the rumen is u (df/dt), and its cumulative proportion until time t is:

$$\begin{aligned}\varnothing_{\text{until}_t} &= \int_0^t u\frac{df}{dt}dt \\ &= \frac{k_p k_c}{k_c - k_p}\int_0^t (r + be^{-k_d t})(e^{-k_p t} - e^{-k_c t})dt \\ &= r\left(1 - \frac{k_c e^{-k_p t} - k_p e^{-k_c t}}{k_c - k_p}\right) + b\frac{k_p k_c}{(k_d + k_p)(k_d + k_c)} \\ &\quad \left(1 - \frac{(k_d + k_c)e^{-(k_d + k_p)t} - (k_d + k_p)e^{-(k_d + k_c)t}}{k_c - k_p}\right)\end{aligned} \quad (5)$$

As time from feeding increase, the fraction of undegraded feed leaving the rumen (u) falls to zero, and so the cumulative flow of undegraded feed DM approaches closer and closer to the RUDM value:

$$\text{RUDM} = r + b\frac{k_p k_c}{(k_d + k_p)(k_d + k_c)} \quad (6)$$

This equation is equivalent to equation (3), which determines RUDM as the complementary value of ruminal degradability.

Finally, the weight proportion of each residue to be included in the composite sample was determined from the flow proportion in each interval. The RUOM and RUCP were determined from the concentrations (DM basis) of the tested fractions in the composite sample (X) and whole feed (Y), and the value of RUDM was obtained by the integration method: RUOM or RUCP = $X \times \text{RUDM}/Y$.

The IED of CP was then determined from its concentration in the composite sample (X) and in the intestinally incubated residues (Z), as well as from the value of IED of DM as: IED of CP = 1 – [$Z\times$ (1 – IED of DM)/X].

Chemical analyses

The tested samples or the incubated residues were analysed in accordance with AOAC (2000) for concentrations of DM (procedure 934.01), ash (procedure 967.05), ether extract (procedure 920.39) and CP (procedure 968.06), using for this last fraction a Leco FP-528 combustion analyzer (Leco Corp., St. Joseph, MI, USA). An Ankom fibre analysis system (Model 220; Ankom Technology Corp., Macedon, NY, USA) was used to determine in sequence neutral detergent fibre (Van Soest et al., 1991), acid detergent fibre and acid detergent lignin (Robertson and Van Soest, 1981). The neutral detergent solution was with alpha-amylase but without sodium sulphite. Neutral and acid detergent fibres were expressed inclusive of residual ash. Insoluble nitrogen in neutral detergent (NDIN) and in acid detergent (ADIN) solutions was determined by N analysis of the respective residues. Samples of duodenal content collected for transit studies were processed as described by González et al. (1998) and analysed for Yb and Eu by atomic absorption or emission spectrometry respectively. Nitrogen isotopic proportions were analysed by mass spectrometry (VG Prism II, IRMS linked in series to a Dumas-style Carlo Erba EA 1108 N analyzer, Milan, Italy).

Statistical methods

The kinetics associated with the different models used were fitted using non-linear regression models. Mean

values of transit variables for SFM and RGH were compared by variance analysis considering wethers as blocks. This same design was used to examine the effect of considering k_c on RUDM, RUOM and RUCP results and to compare results of RUCP by both methods. Results obtained with the proposed method for RUDM, RUOM, RUCP and IED of DM and CP were studied by variance analysis with a split-plot treatment arrangement. In this design, the values generated using either k_p or both k_c and k_p were the whole plot, which was tested against the wether × transit model interaction as error term, and microbial correction (uncorrected vs. corrected values) and its interaction with the transit model were the subplot treatments. All analyses used the Statistical Analysis System for Windows software, SAS version 6.12.

Results

Transit studies

Concentrations of markers of both feeds fitted well to the model. Thus, R^2 values ranged from 0.989 to 0.997 in SFM and from 0.977 to 0.997 in RGH. The mean value of k_c was lower ($p < 0.05$) in RGH than in SFM, whereas there was no difference between feeds for k_p (Table 2). Values of k_c had a higher variability in SFM than in RGH. In this way, SE were approximately 6.5 times higher (0.0913 vs. 0.0142). A tendency ($p = 0.082$) to a higher time of marker appearance occurred for RGH. As a consequence of these variations, especially that of k_c, the mean retention time in the forestomachs tended ($p = 0.077$) to be higher for RGH vs. SFM.

In situ studies

Mean DM solubility was similar to DM disappearance at 0 h in SFM, whereas a higher DM solubility ($p < 0.05$) occurred in RGH (Fig. 1).

Table 2 Transit through the forestomachs of sunflower meal and Italian ryegrass hay particles

Transit variables*	Sunflower meal	Italian ryegrass hay	SEM	p
k_p (/h)	0.0623	0.0609	0.0041	0.830
k_c (/h)	0.5766	0.0892	0.0652	0.034
Time of marker appearance (h)	1.12	1.24	0.025	0.082
Mean retention time† (h)	19.1	29.9	2.25	0.077

*k_p, rumen particulate passage rate; k_c, rate of comminution and mixing of particles.

†($1/k_p + 1/k_c$ + time of marker appearance at the duodenum).

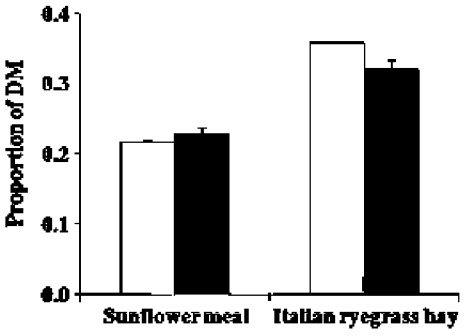

Fig. 1 Comparison of DM solubility (□) and 0-h disappearance (■) values in sunflower meal (SFM; $p = 0.209$) and Italian ryegrass hay (RGH; $p < 0.05$). Bars are SE.

When k_c values were taken into account, changes occurred in the residue proportions needed to compose representative samples of the feed rumen outflow: the residue contribution needed was reduced for the first incubation times followed by an increase starting from the four (SFM) or 12 h (RGH) residues (Fig. 2).

Using the integration method and both k_p and k_c, the uncorrected values of RUDM were lower in both feeds than estimates using only k_p ($p < 0.01$; Table 3). This effect also occurred for RUCP with this same method ($p < 0.01$ for SFM and $p < 0.05$ for RGH). However, the proposed method had a high variability, and as a consequence, this difference was only a tendency ($p = 0.086$) in SFM (Table 3). No differences in RUCP estimates between methods occurred, except for a tendency ($p = 0.090$) to be higher in the integration method using k_c and k_p values in SFM (Table 3). When k_c values were used in the model, the microbial contamination in the DM, OM and CP of the composite samples increased (Table 4). These increases were very large and significant ($p < 0.05$) in RGH and small and not significant in SFM.

The ^{15}N abundance (atoms %) in the water-insoluble fraction (0-h incubation bags) of SFM and RGH was the same at 0.369. The average of this parameter in the SAB samples was 0.458 (SE = 0.0162), whereas concentrations of OM and CP (g/kg DM) were 843.3 (SE = 0.57) and 501.9 (SE = 0.52). The correction for microbial contamination led, in both feeds, to decreases in RUDM, RUOM and RUCP, as well as in the IED of DM and CP (Table 5). The incorporation of k_c in the model also led to decreases in all these values, although for CP in SFM the decrease was only a tendency ($p = 0.086$ for RUCP and $p = 0.052$ for its IED), as a consequence of the

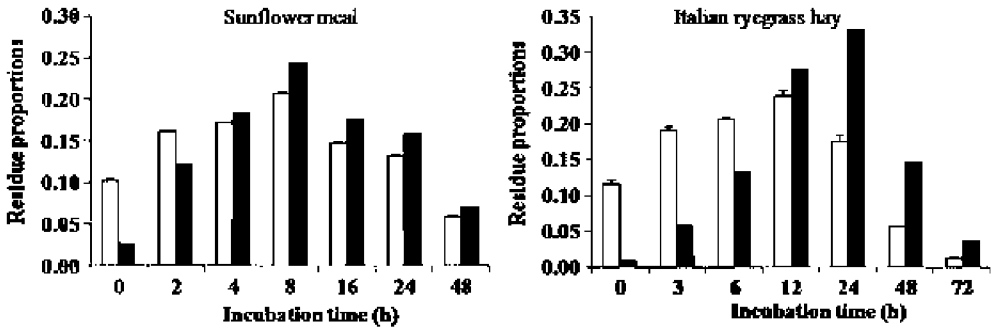

Fig. 2 Effects of considering only the rumen passage rate (k_p, □) or this rate together with feed particle comminution (k_c, ■) on the residue proportions needed to composite representative samples of ruminal DM outflow from sunflower meal (SFM) or Italian ryegrass hay (RGH). Bars are SED values. Differences were significant at $p < 0.01$, except for samples at 4 h ($p < 0.05$) in SFM, and at 12 h ($p = 0.079$) and at 48 h ($p < 0.05$) in RGH.

Table 3 Dry matter (DM) and crude protein (CP) degradation kinetics, effects of using the particle comminution rate (k_c) in addition to the rumen passage rate (k_p) in non-corrected estimates of the rumen undegraded fraction and comparison of results from integration and proposed methods

	Sunflower meal					Italian ryegrass hay				
	DM	CP				DM	CP			
	Integration method	Integration method	Proposed method	SEM	p*	Integration method	Integration method	Proposed method	SEM	p*
Degradation kinetics										
a†	0.235	0.280				0.320	0.160			
b	0.460	0.662				0.426	0.445			
r	0.305	0.0577				0.255	0.395			
k_d (/h)	0.172	0.230				0.0454	0.0875			
Ruminally undegraded fraction										
Using k_p	0.431	0.204	0.192	0.0077	0.398	0.498	0.578	0.587	0.0067	0.437
Using k_c–k_p	0.404	0.165	0.154	0.0026	0.090	0.415	0.486	0.497	0.0154	0.676
SEM	0.0009	0.0019	0.0086			0.0057	0.0068	0.0271		
p‡	0.002	0.005	0.086			0.009	0.011	0.145		

*Effect of *in situ* method.
†*a*, *b* and *r* represent soluble, non-soluble degradable and undegradable fractions, respectively. k_d, fractional degradation rate of fraction *b*.
‡Effect of transit model.

Table 4 Effect of the use of the particle comminution rate (k_c) in addition to the rumen passage rate (k_p) on the microbial fraction included in the representative samples of rumen undegraded outflow

	Sunflower meal				Italian ryegrass hay			
	k_p	k_c, k_p	SEM	p	k_p	k_c, k_p	SEM	p
Dry matter	0.0047	0.0055	0.00039	0.308	0.0692	0.108	0.00620	0.048
Organic matter	0.0041	0.0048	0.00034	0.309	0.0610	0.0951	0.00543	0.047
Crude protein	0.0170	0.0208	0.00171	0.263	0.463	0.706	0.0195	0.013

higher variability of both the proposed technique and the SE of k_c in this feed. Interactions between contamination correction and transit model ($p < 0.05$) only occurred for the IED of DM and CP in RGH (Table 5).

Discussion

Feed transit kinetics

With samples obtained from the duodenum, the large differences observed between hay and concentrate

Table 5 Effects of the use of the particle comminution rate (k_c) in addition to the rumen passage rate (k_p) and the correction of microbial contamination in *in situ* feed evaluation

	k_p		k_c, k_p		Transit model		Microbial correction	
Item	NC*	C	NC	C	SEM	p	SEM	p
Sunflower meal								
RUDM	0.431	0.429	0.404	0.402	0.0008	0.002	0.0002	<0.001
RUOM	0.445	0.443	0.417	0.415	0.0009	0.002	0.0002	<0.001
RUCP	0.192	0.189	0.154	0.150	0.0086	0.086	0.0002	<0.001
IED of DM	0.217	0.214	0.183	0.180	0.0023	0.009	0.0003	<0.001
IED of CP	0.752	0.750	0.699	0.697	0.0089	0.052	0.0005	0.047
Intestinal digested								
DM	0.0940	0.0922	0.0740	0.0723	0.0016	0.013	0.0001	<0.001
CP	0.146	0.143	0.107	0.105	0.0091	0.098	0.00002	<0.001
Italian ryegrass hay								
RUDM	0.498	0.464	0.415	0.371	0.0043	0.005	0.0015	<0.001
RUOM	0.518	0.487	0.432	0.391	0.0046	0.005	0.0013	<0.001
RUCP	0.587	0.316	0.497	0.143	0.0133	0.026	0.0153	<0.001
IED of DM†	0.112	0.0609	0.0786	0.0014	0.0062	0.033	0.0015	<0.001
IED of CP‡	0.636	0.494	0.598	0.306	0.0045	0.003	0.0093	<0.001
Intestinal digested								
DM	0.0561	0.0286	0.0327	0.0005	0.0037	0.039	0.0008	<0.001
CP	0.373	0.156	0.297	0.0433	0.0082	0.015	0.0049	<0.001

RUDM, RUOM and RUCP, ruminally undegraded dry matter, organic matter and crude protein, respectively; IED, intestinal effective digestibility.
*Microbial contamination: NC = not corrected; C = corrected.
†Significant interaction: SEM = 0.00209; $p < 0.01$.
‡Significant interaction: SEM = 0.0131; $p < 0.01$.

particles in the highest fractional rate constant could only occur in the rumen. This agrees with Ellis et al. (1979) and González et al. (2006b), who pointed out that this rate should be ascribed to an intraruminal compartment associated with the process of density increase and comminution and mixing of particles (k_c) needed for their escape from the rumen trapping mechanisms, as sequestration in the raft or filtering effects in passage from the rumen ventral sac to reticulum. This process took longer in RGH than in SFM owing to the higher initial size and the slower comminution (by rumination and fermentation) of forage particles, and also because forages have initial values of specific gravity lower than concentrates. Both particle size reduction and specific gravity rise have been considered as main factors affecting particle residence time in the rumen (Poncet, 1991). Differences on the outflow of fine particles from the reticulum corresponding to the k_p values may not show large differences between forages and concentrates, because the outflow of fine particles through the reticulo-omasal orifice is mainly controlled by diet conditions (e.g. intake level, forage proportion) and the animal's physiological status (Poncet, 1991). Using duodenal sampling and mixed diets with forage to concentrate ratio varying from 90:10 to 40:60 supplied to wethers *ad libitum* or at 1.1 the energy maintenance level, Poncet et al. (1987) did not found differences for k_p between dactyl hay, dehydrated lucerne and soybean meal. Also, values of first marker appearance time (usually associated with retention time in other compartments) were similar to the time spent in the abomasum (Warner, 1981). The proportion of total residence time in the reticulo-rumen associated with the k_c rate was large in RGH (40.5%) and moderate in SFM (9.6%). This process always determines a delay in particle escape, and therefore, k_c should be considered in addition to k_p to improve accuracy of *in situ* estimates.

In situ estimates

The similar, or close, uncorrected estimates of RUCP obtained with both methods agree with results obtained by our group using only k_p (González et al., 2006a, 2009; Arroyo et al., 2009). The proposed method has advantages compared to mathematical integration-based methods to obtain effective estimates of both ruminally undegraded fractions and intestinal digestibility: (i) increasing feed fractions simultaneously tested owing to the larger available

samples, (ii) large reductions in the number of samples to incubate in the intestine, in analyses derived from ruminal and intestinal incubations and in the kinetics to be fitted and (iii) simplified microbial contamination correction of estimates of ruminally undegraded fractions and IED.

Most limitations usually addressed to *in situ* methodologies cannot be considered in this experiment. Thus, the similar results of DM solubility and 0-h disappearance from the bags seem to show that particle losses would be very low in both tested feeds. Also, the assumption (usual in *in situ*-based CP evaluation systems) that soluble CP is totally degraded in the rumen has been questioned based on *in vitro* and *in vivo* assays (Choi et al., 2002; Hedqvist and Udén, 2006). However, these works are not demonstrative as a consequence of design limitations.

The interest in considering the k_c rate to obtain more reliable estimates of ruminally undegraded fractions was underlined by the Agricultural Research Council (1984), but this approach has been rarely employed, as *in situ* methods have been usually considered simplistically, because simplicity is an advantage for systematic evaluation studies. Present results support previous comments that not considering k_c implies large changes of rumen escape estimates in most feeds (González et al., 2006b). In addition, present results show that the interest to consider k_c is not only associated with the increased estimations about the rumen residence time of feed particles but also with its effects on microbial contamination of the samples representing the feed rumen outflow and on the IED of these samples. The increased microbial contamination when using k_c is because of the exponential rise in this proportional contamination associated with increased rumen residence time (González et al., 1998; Krawielitzki et al., 2006; Rodríguez and González, 2006) and of the increased contribution to the composite samples of residues incubated for longer times. Therefore, this contamination and its increase using k_c vary with feeds.

Intestinal digestibility is usually studied using mobile bags at a fixed rumen incubation time. However, as the undegraded feed outflow is a time continuous process after the feed ingestion, this procedure cannot be considered physiological. Therefore, estimates considering this continuous outflow should result in more accurate intestinal digestibility values. Thus, decreased IED estimates when k_c is used are in agreement with the increase in the proportion of undigestible feed compounds associated with the extent of degradative actions with increased rumen residence time (González et al., 1999). As adherent micro-organisms are mostly digested during intestinal incubation, the lack of correction of the microbial contamination in the rumen is also associated with IED overestimations, as shown by Arroyo et al. (2009) and González et al. (2009). Results agree with the observations of these latter authors that the IED error increases with the contamination level and differences in the intestinal disappearance between SAB and the tested feed. Rodríguez et al. (2008) also showed low microbial contamination for SFM. This contamination is always high in forages, especially in grasses (Bernard et al., 1988; Rodríguez and González, 2006; Kamoun et al., 2007). In contrast, disappearance of SAB from bags during intestinal incubation seems to be high and similar in concentrates and forages (97.1 and 94.2% in SFM, and 97.6 and 98.3% in RGH disappear considering either only k_p or this rate together with k_c respectively). Therefore, the difference in intestinal disappearance between feed and SAB should have a larger effect on the IED error in forages than in concentrates, as shown in our study.

Criticisms to the mobile bag technique have been made because intestinal absorption may be lower than bag disappearance, which leads to overestimate the intestinal digestibility. However, considering the scarce possibility of physical losses of particles after the process of ruminal incubation and bag washing as well as the low values of IED showed for SFM and especially for RGH, this error would be small. In addition, when the recovery of bags is made from the faeces, the microbial adherence to feed particles in the hind-gut may underevaluate these values. However, this contamination seems to be also low (Yang and Poncet, 1988; Jarosz et al., 1994). Therefore, compensation, at least partial, may be expected for these both errors.

Because errors about the rumen-undegraded fraction and its IED are always overestimations, methods omitting k_c or contamination correction overestimate true values of the intestinal digestible fraction. As shown in present and previous results (González et al., 2006b), both sources contribute to large cumulative errors in forages, whereas not using k_c is the most important source of error in concentrates like SFM.

The content of microbial corrected RUCP of RGH considering k_c (0.143) was lower than PDI (Vérite, 1987) and NRC (2001) values (0.340 and 0.292 respectively) proposed from studies lacking of k_c and microbial correction. The associated value of IED (0.306) was also lower than those proposed by these

systems (0.70 and 0.65 respectively). These low values agree with results of green and ensiled Italian rye-grass (González et al., 2007) showing that the protein value in grass forages is mainly determined by their potential to promote microbial protein synthesis in the rumen. Thus, based on the true value of effective degradability of OM when k_c and k_p are considered (0.609), as well as in the specifications for this synthesis of the PDI system (145 g microbial CP/kg ruminal fermented OM and a proportion of 0.64 intestinal digested true protein), the corrected supply of digestible rumen-undegraded protein of RGH represents only a proportion of 0.05 of the metabolizable protein supply.

The corrected estimate of RUCP in SFM obtained using both k_p and k_c (0.150) is lower than the value proposed in the PDI system (0.230) but similar to that proposed by the NRC (0.134). The associated mean value of IED of CP (0.697) is however lower than values established by the PDI and the NRC systems (0.85 or 0.90 respectively).

Conclusion

This study shows that not considering k_c and/or not correcting for the microbial contamination of particles which takes place in the rumen results in different estimates of the ruminally undegraded fraction and its intestinal digestibility. Because both corrections are cumulative for both parameters, the final error in the intestinally digested CP is very large in RGH as well as in SFM. This fact highlights the need to conduct *in situ* studies in these conditions to obtain accurate results. The developed mathematical model for the cumulative flow of rumen-undegraded feed and the proposed *in situ* procedure allows these estimates to be obtained for multiple feed fractions with an important reduction in efforts and costs.

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